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REVIEW

Engineering plant oils as high-value industrial feedstocks for biorefining: the need for underpinning cell biology research

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Plant oils represent renewable sources of long-chain hydrocarbons that can be used as both fuel and chemical feedstocks, and genetic engineering offers an opportunity to create further high-value specialty oils for specific industrial uses. While many genes have been identified for the production of industrially important fatty acids, expression of these genes in transgenic plants has routinely resulted in a low accumulation of the desired fatty acids, indicating that significantly more knowledge of seed oil production is required before any future rational engineering designs are attempted. Here, we provide an overview of the cellular features of fatty acid desaturases, the so-called diverged desaturases, and diacylglycerol acyltransferases, three sets of enzymes that play a central role in determining the types and amounts of fatty acids that are present in seed oil, and as such, the final application and value of the oil. Recent studies of the intracellular trafficking, assembly and regulation of these enzymes have provided new insights to the mechanisms of storage oil production, and suggest that the compartmentalization of enzyme activities within specific regions or subdomains of the ER may be essential for both the synthesis of novel fatty acid structures and the channeling of these important fatty acids into seed storage oils.

Introduction

The production of industrially important oils in oilseed crops is a major goal of the plant biotechnology community and represents an important step in our transition from a crude oil-based society to a more sustainable, biobased economy. The 'bioeconomy' refers to all economic activity derived from (1) the development of a better understanding of the underlying molecular mechanisms of biological systems and (2) the application of this knowledge to expand and diversify products obtained from agricultural, health-related, chemical or energy industries. Toward this end, biorefining aims to fractionate and capture valuable feedstocks from plants that can be used as sources of energy and/or chemical feedstocks for industry. However, unlike traditional oil refining, which is based on non-renewable petroleum, biorefining is an entirely renewable process that takes advantage of the robust biosynthetic machinery of plants that, driven by photosynthesis, produces a diverse array of compounds that can be used to support human activities.

While many of the genes involved in plant lipid metabolism have been identified and powerful analytical tools are readily available for quantifying lipid products, a significant void exists in our understanding of the cell

Abbreviations – DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FAD, fatty acid desaturase; GFP, green fluorescent protein; MMT, million metric tons; PDAT, phospholipid:diacylglyceral acyltransferase; SCD, stearoyl-CoA desaturase; TAG, triacylglycerol; TMD, transmembrane domain.

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biological processes that take place between the synthesis of the encoded gene products and the production of their respective lipid metabolites. As a result, any future rational design of crop plants to produce specific types of industrial oils will benefit significantly from a better understanding of the assembly and regulation of lipid-modifying enzymes.

In this review, we briefly describe the current dependence of society on crude oil as a source of both fuel and chemical feedstocks and the potential of using plants as alternative, sustainable sources of energy and raw materials for industry. We then describe the state of the art in terms of our ability to produce renewable, industrially important oils in plants and the need for a better understanding of the molecular and cellular events that will underpin rational engineering design. Last, we provide an overview of the cellular properties of several sets of enzymes that are critically important for determining the composition of seed storage oils including fatty acid desaturases (FADs), divergent FADs, which are capable of producing many different types of industrially important fatty acids, and diacylglycerol acyltransferases (DGATs), which transfer fatty acids into triacylglycerols (TAGs). Recent results obtained with plant DGAT enzymes, as well as emerging evidence from mammals, suggest that compartmentalization of enzyme activities may be essential for the synthesis and channeling of fatty acids into storage oils. The implications for producing high-value industrial oils in plants are discussed.

Modern society's dependence on crude oil and the potential of plants as alternative, renewable sources of energy and chemical feedstocks

In our modern era, crude oil underpins all aspects of society. For example, the global annual consumption of crude oil in the year 2005 was approximately 4200 million metric tons (MMT), which translates to about 85 million barrels of oil per day. Almost 90% of this oil is used as fuel that supports the transportation and energy sectors, while the remaining 10% is used by the petrochemical industry for the production of plastics, fibers and other components fabricated for items used in our daily lives (Fig. 1).

The impact of crude oil on so many aspects of society is due to its relatively cheap price and the rich chemical composition of the oil itself. Crude oil is a complex mixture of many different hydrocarbons and chemicals that first has to be fractionated into its component parts before they can be used. For instance, during the refining process, crude oil is heated and the resulting vapors gradually cool as they travel up a distilling tower. During this process, hydrocarbons of similar chain length precipitate out and are collected into fractions including bottled gases (e.g. methane, ethane, propane and butane), gasoline, kerosene, diesel, as well as the longest chain hydrocarbons like lubricants, waxes, tar and coke. Because the gasoline fraction is of the highest demand, numerous methods have been developed to convert the longer chain hydrocarbons into shorter chains. Collectively, these processes are referred to as 'cracking and reforming' and, in addition to gasoline, yield a variety of by-products, including alkenes and aromatics, that serve as the primary feedstocks for the petrochemical industry.

Overall, the utilization of crude oil for the production of both fuel and industrial feedstocks has been arguably one of the most successful economic endeavors in modern history. Indeed, there are a number of important lessons learned from the crude oil scenario that can be applied to the emerging biobased economy. For instance, the success of crude oil utilization can be attributed to several factors including having an inexpensive, but complex starting material (petroleum), which is used in a variety of different ways, thus providing for multiple marketing opportunities. The petrochemical industry has been also highly successful in further diversifying the products derived from crude oil, with most of these products being low cost and high quality, which are both important determinants for economic success. But while the refining and subsequent utilization of crude oil has played a major role in the development and advancement of society, decreases in oil production, volatility of pricing, increasing environmental concerns and the need to develop a domestically secure source of energy and chemical feedstocks, has led many countries to explore alternative sources of fuels and industrial raw materials.

Plants are well positioned to help meet society's need as an alternative to crude oil because they are renewable (i.e. a portion of the harvested seeds can be used to regenerate the entire process), and the majority of plant biomass is housed within their cell walls, which is an excellent potential source of fuel (Fig. 1). Likewise, seeds are ideal biofactories for the production of valuable feedstocks including oils, protein and starch, many of which are already used for fuel or industrial purposes (Fig. 1) (Metzger and Bornscheuer 2006, Scheller and Conrad 2005). Combined with these positive attributes, biotechnology, like the petrochemical industry, can be used to greatly expand the number and types of products that can be derived from plant sources. Therefore, the overall model for biorefining is fairly similar to that of petroleum refining (Fig. 1), but biorefining has the added advantage of being more environmentally friendly and represent a renewable, sustainable source of fuels and chemical feedstocks for industry. The major challenge

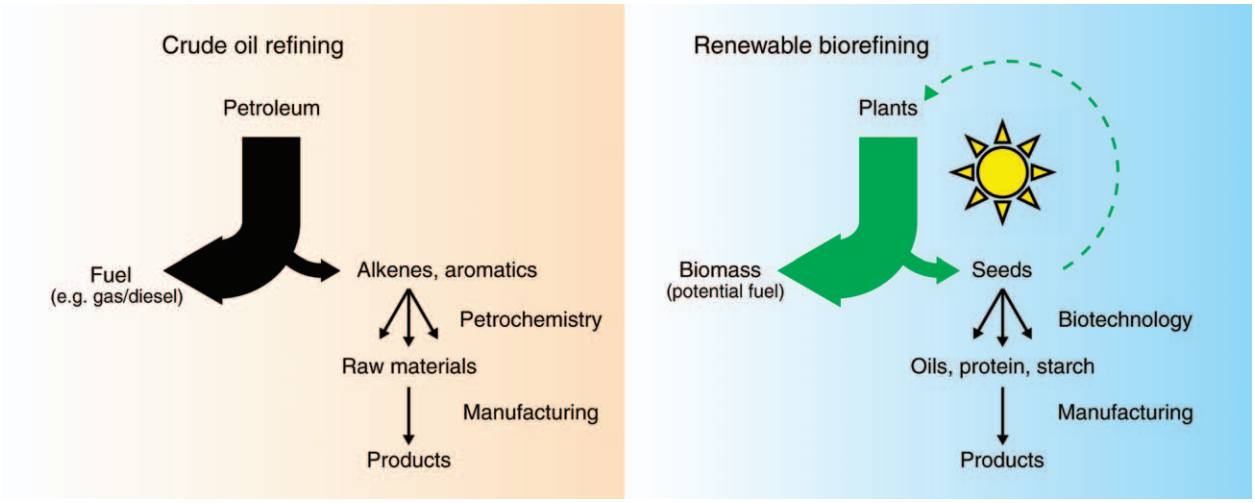

Fig. 1. Comparison of traditional oil refining to the emerging, renewable biorefining model. Each scenario is similar and includes a relatively cheap, but complex starting material (petroleum or plants) is fractionated into component parts for usage in a variety of industries including energy production (i.e. fuels) and manufacturing. The size of the arrows during this initial fractionation process represents the relative amounts of carbon that are partitioned into the respective industries. As illustrated, the petrochemical industry in the left panel plays a key role in generating a more diverse array of raw materials that can be subsequently used by manufacturers to produce new and value-added products. Similarly, biotechnology (right panel) is expected to be important for generating higher value feedstocks for production of bioproducts, which will offset the lower profits obtained from biofuels. Unlike typical crude oil refining, which is based on a diminishing starting material (petroleum) that contains hydrocabons derived from the energy of 'ancient sunlight' (i.e. solar energy originally captured by photosynthetic organisms and then stored in the form of reduced carbons), biorefining is entirely renewable and sustainable, driven by repeated rounds of photosynthetic energy capture, biochemical diversification through metabolic activity, harvesting and fractionation and subsequent replanting of a portion of the seeds to replenish the overall process. Plants, therefore, represent renewable, sustainable sources of energy and raw materials that can help support human activities in an emerging bioeconomy.

faced at the moment, however, is how to implement the biorefining process from an economically feasible standpoint. Fuel and feedstocks derived from plants must be cost competitive with their crude oil counterparts to be adopted by industry and society. To accomplish this, there is clearly a need for additional research to engineer plants to produce optimized, high-value feedstocks that can be readily recovered by cost-effective extraction and fractionation processes.

The overall economic success of biorefining will depend in part on the ability to produce high-value traits in plants that can offset smaller gains obtained in other areas. For example, the production of fuel ethanol from sugar is an energy-intensive process that, despite current subsidies, results in a relatively small profit margin. The economics of this process could be improved significantly by generating, through various biotechnology strategies, greater values from other parts of the plant, for example, value-added seed oils or biopolymers. Such genetic engineering of plant seeds for production of industrial oils, or other value-added oils such as 'fish oil'–type fatty acids, would provide not only renewable sources of these important feedstocks to industry but also new economic incentives for the continued development of a biorefining model in our modern society.

Plant oils as industrial feedstocks

It has long been recognized that the seed oils of plants are chemically similar to the long-chain hydrocarbons from crude oil and, thus, represent outstanding renewable sources of fuel and raw materials for industry. As mentioned above, benefits from the production of industrial oils in plants include a decreased dependence on foreign crude oil imports, novel chemical feedstocks that are not easy to produce by traditional synthetic routes and expansion of the competitiveness and viability of the agricultural sector of society.

There is already a significant amount of vegetable oil that is used for industrial purposes. For example, oilseed crops are major agricultural commodities, with more than 380 MMT of oilseeds produced in 2005. About 85% of extracted vegetable oil is used for food and cooking applications, while nearly 15% is used for industry including lubricants, inks, coatings, plasticizers and biodiesel. However, one significant problem encountered when using vegetable oils for industrial purposes is that the fatty acid components are not especially reactive.

That is, because oilseed crops have been domesticated as a food source and not for industry, their fatty acid composition is tailored primarily toward the needs of human nutrition. Seed oils, therefore, have to be chemically treated before they can be used for the production of lubricants, plastics and so forth, and this contributes significantly to the costs and environmental impact associated with their utilization (Metzger and Bornscheuer 2006).

What is particularly attractive to plant biotechnologists today is the understanding that many non-domesticated plant species contain hundreds of structurally different fatty acids in their oils, and many of these fatty acids have direct uses in industry (Smith 1971). Importantly, the majority of these oils are enriched in a single 'unusual' fatty acid (e.g. up to 90% of seed oil fatty acid composition), which simplifies downstream processing and, in some cases, allows for direct usage of the oils in industrial applications. For example, some plants such as tung (*Vernicia fordii*) and marigold (*Calendula officinalis*) accumulate high amounts of conjugated fatty acids that are easily oxidized and, therefore, make excellent ingredients in formulations of inks, dyes, coatings and resins (Sonntag, 1979). Other plants such as castor (*Ricinus communis*) or *Lesquerella fendleri* accumulate hydroxy fatty acids that can serve as lubricants, sealants and surfactants (Metzger and Bornscheuer 2006). Seeds of *Crepis alpina* contain high amounts of epoxy fatty acids that could find widespread usage as plasticizers and adhesives and could be chemically cleaved to produce monomeric components of nylon. Some plants such as the desert shrub jojoba (*Simmondsia chinensis*) even contain fatty acids in the form of liquid waxes that tend to perform well in high temperature and pressure applications such as engine lubricants and hydraulic fluids. In addition to these types of fatty acids, other fatty acids derived from plant oils may have combinations of functional groups (Cahoon and Kinney 2004), which are typically difficult to prepare by traditional synthetic chemistry and petroleum-based feedstocks and, thus, may serve as unique starting materials for novel products.

Unfortunately, the central problem with using these seed oils for industrial purposes is that the plant species that produce these industrially important oils generally have limiting agronomic traits such as small seeds, low yields, toxicity and/or may have very limited geographical growing areas. Therefore, it is difficult to obtain enough of the seed oils, in a cost-competitive manner, for their usage in industry. As a result, a major goal of the plant biotechnology community over the past several years has been to study these wild plants to isolate genes for fatty acid biosynthesis, then transfer these genes to high-yielding crop plants. Indeed, this agenda has been aggressively pursued by many industrial, academic and government scientists, resulting in the identification of many of the genes for exotic fatty acid biosynthesis (Cahoon et al. 2007, Dyer and Mullen 2005, Napier 2007). The expression of these genes in transgenic plants, however, has consistently resulted in a much lower than expected accumulation of the desired fatty acid in comparison with the amount observed in the native plants (reviewed in Drexler et al. 2003, Thelen and Ohlrogge 2002). For example, ricinoleic acid is a hydroxylated fatty acid that accumulates to about 90% in the seed oil of castor bean, but expression of the castor hydroxylase gene in *Arabidopsis thaliana* results in accumulation of only 17% ricinoleate. Similarly, expression of the gene in *Brassica napus*, a true oilseed crop, results in accumulation of only 16% ricinoleate. Expression of other genes responsible for the synthesis of epoxy, acetylenic or conjugated fatty acids in transgenic plants has resulted also in low accumulation of the desired fatty acid product. These low yields may be because of a variety of reasons including suboptimal transgene expression, low enzyme activity or rapid protein turnover, improper subcellular localization, poor substrate availability, improper exclusion of fatty acid products from cellular membranes and/or inefficient incorporation of fatty acids into seed storage oils. Whatever the reason, it is now clear that multiple genes will be required for the efficient synthesis and accumulation of exotic fatty acids in transgenic plants and that there is a pressing need to develop a better understanding of how these exotic fatty acids are eventually channeled into storage oils.

Biosynthesis of tung oil as a model system for understanding the production of valuable industrial oils in plant seeds

To address the above-mentioned challenges, we have been studying the tung tree as a model for production of seed oil containing high amounts of an industrially important fatty acid. Tung is a subtropical tree that produces fruit annually and the seed oil is used as a drying agent in the formulations of inks, dyes, coatings and resins (Sonntag, 1979). The unique drying qualities of tung oil are determined by its fatty acid composition, which is dominated by a single unusual fatty acid called eleostearic acid (approximately 80% of total fatty acid composition). Eleostearic acid has three conjugated double bonds at the 9, 11 and 13 positions of the 18-carbon fatty acid chain, and because conjugated double bonds are easily oxidized, spreading a thin layer of tung oil on a surface results in the rapid oxidation and polymerization of eleostearic acid and, thus, the formation of a protective, waterproof barrier. The tung tree itself has limited

agronomic characteristics, and, therefore, the price and availability of the oil can fluctuate quite dramatically (e.g. \$0.50–\$1.00 per pound). Soybean oil, in contrast, is significantly less expensive and quite easy to produce, costing only \$0.15–\$0.20 per pound. Consequently, a major goal is to produce tung-like drying oils in transgenic crops such as soybean to provide a more reliable, potentially cheaper source of drying oil to industry; however, to do this, more information is needed about how the process of tung oil biosynthesis occurs.

As shown in Fig. 2, tung oil biosynthesis, similar to the production of oils in other developing seeds, begins with the synthesis of basic fatty acids in the plastid, with one of the main products being exported as oleoyl-CoA. The oleic acid is subsequently transferred to the phospholipid fraction in the ER, where two enzyme activities are present to produce eleostearic acid. First, FAD2 desaturates oleic acid ($18{:}1\Delta^{9}$) to produce linoleic acid ($18{:}2\Delta^{9,12}$), then a so-called diverged FAD2, referred to as fatty acid conjugase (FADX), converts linoleic acid to eleostearic acid ($18{:}3\Delta^{9,11,13}$) (Dyer et al. 2002). Once the eleostearic fatty acids are made, they are shuttled by a series of complex acyltransferase reactions from the phospholipid fraction to TAG. The TAGs are thought to accumulate in the ER and eventually form a bulge that, with the involvement of various protein components such as oleosins, pinches off from the ER to form a cytoplasmic oil droplet or oil body (Huang 1996, Napier et al. 1996). TAGs in tung oil also contain a small amount of linolenic acid ($18{:}3\Delta^{9,12,15}$), which is produced in plants by an ER-localized enzyme called FAD3. Overall, many of the genes in this pathway have been cloned and their protein products well characterized (Dyer et al. 2002, 2004, Hwang et al. 2004, Shockey et al. 2005, 2006); however, in this review, we will focus only on FAD2, FAD3 and FADX, which synthesize the polyunsaturated fatty acids present within tung oil, and DGAT, which catalyzes the committed step in oil biosynthesis, that is, the formation of TAG.

Subcellular localization and assembly of FADs in the ER of plant cells

Early cloning of FAD2 and FAD3 genes revealed that these enzymes shared significant sequence identity including

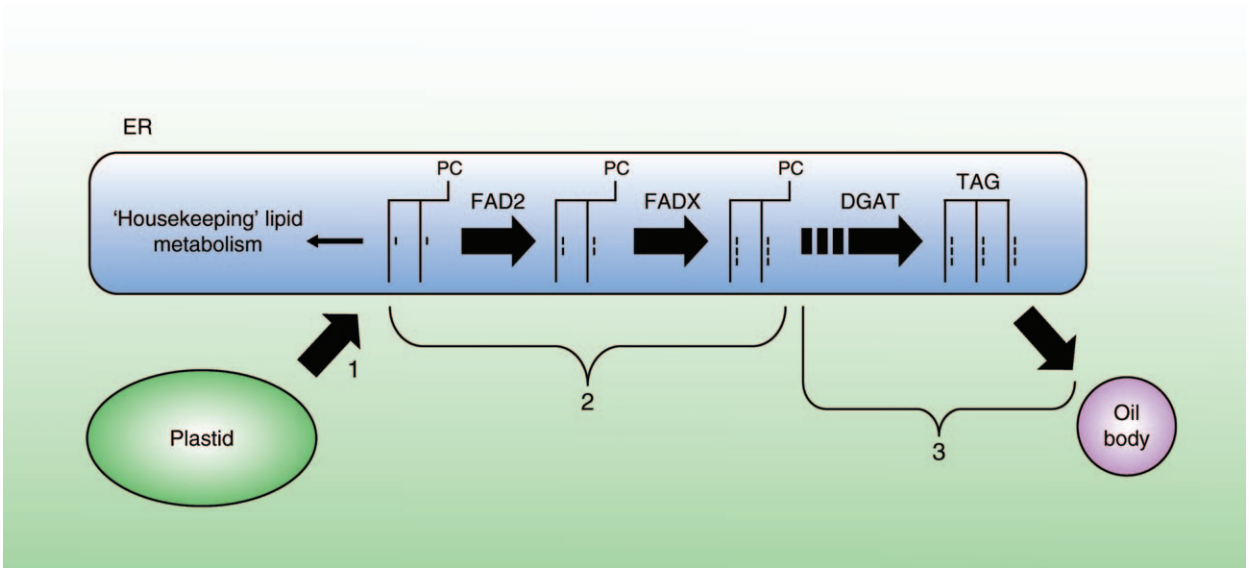

Fig. 2. Diagram representing the major metabolic steps and relative carbon flux during the production of storage oils containing high amounts of industrially important fatty acids. Depicted are three major biosynthetic events that occur during the production of storage oils in all developing plant seeds including: (1) the synthesis of basic fatty acid structures in the plastid and their export to the ER; (2) modification of these basic fatty acid structures by ER-localized FADs and diverged FADs (e.g. the production of eleostearic acid in tung by FAD2 and the diverged FAD2 enzyme FADX) and (3) the packaging of fatty acids into TAG, which accumulate in oil bodies that pinch off from the ER into the cytosol. The extended arrow beneath DGAT, which catalyzes the committed step in TAG biosynthesis by transferring a fatty acid from the fatty acyl-CoA pool to the 3rd position of diacylglycerol to form TAG, represents a complex series of acyltransferase reactions that, along with DGAT enzyme activity, determines the final fatty acid composition of storage oils (Napier 2007). Each of the major metabolic steps in oil biosynthesis occurs in a highly integrated, cooperative fashion, resulting in a massive flux of carbon into storage oil. Disruption of any of these pathways (e.g. poor incorporation of unusual fatty acids into storage oils of engineered plants) can result in decreased metabolic flux of fatty acids into oil, increased degradation of fatty acids in peroxisomes (resulting in futile cycling of fatty acids though repeated rounds of synthesis and degradation; Eccleston and Ohlrogge 1998) and/or reduced yield of desired fatty acids in storage oil. Furthermore, accumulation of unusual fatty acids in membrane lipids may interfere with so-called normal 'housekeeping' functions of the ER (or other organelles of the secretory pathway), leading to general cellular dysfunction and poor agronomic performance of transgenic seeds.

a number of equally spaced transmembrane domains (TMDs) and three conserved histidine-enriched regions thought to coordinate di-iron atoms at the active site center (Fig. 3A) (Shanklin and Cahoon 1998). Biochemical analysis and immunolocalization studies of FAD2 and FAD3 showed also that these enzymes are located exclusively in the ER, and, furthermore, that they adopt a similar topological orientation in ER membranes in which their N- and C-termini are exposed to the cytosol (Fig. 3A) (Dyer and Mullen 2001 and references therein). Further investigations of the ER insertion mechanism for FAD2 and FAD3 revealed that they insert cotranslationally, and not post-translationally, into ER membranes, with their first TMD likely serving as a signal-anchor sequence (McCartney et al. 2004).

Alignments of several FAD2 and FAD3 proteins, including tung FADX, revealed that FAD3 proteins often contained a C-terminal dilysine ER retrieval motif

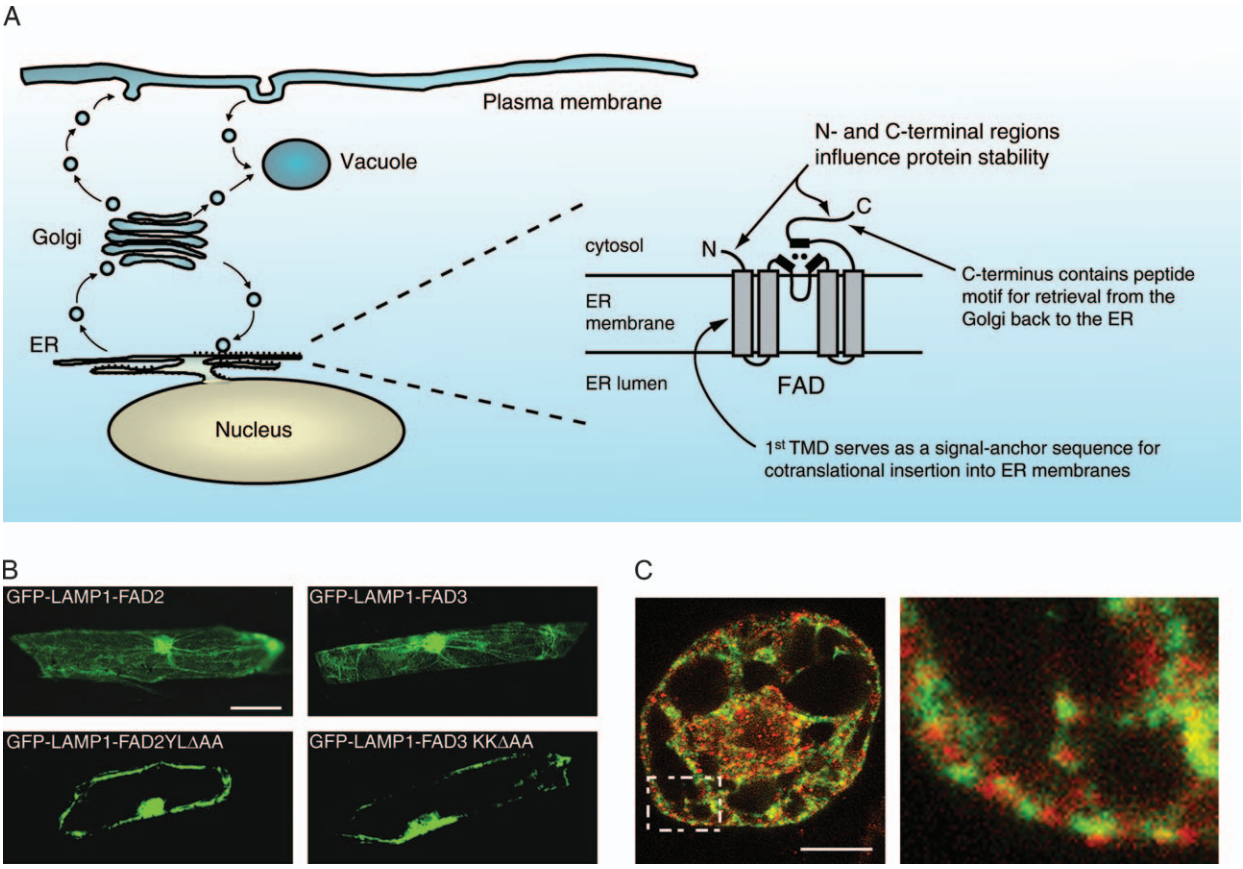

Fig. 3. Cell biology of enzymes involved in storage oil production in developing seeds. (A) Cartoon depicting the ER as a key component of the secretory pathway of the plant cell. FADs are ER-resident membrane proteins that contain a variety of molecular signals responsible for either their initial targeting to the ER by cotranslational insertion, steady-state maintenance in the ER through retrieval from post-ER compartments (e.g. Golgi), or determination of the protein's half-life (see main text for additional details). The model shown for FAD protein structure, including positions of the predicted membrane-spanning domains and orientation of the N- and C-termini, histidine boxes and di-iron active site, is based on previous studies (Dyer and Mullen 2001, Shanklin et al. 1994). (B) Identification of ER retrieval signals in FAD2 and FAD3 proteins. Shown are fluorescence micrographs of onion epidermal peels bombarded with DNA constructs encoding a membrane-bound reporter protein [consisting of GFP appended to the C-terminal portion of lysosomal membrane-associated protein (LAMP1), fused to C-terminal peptide sequences derived from either FAD2 (GFP-LAMP1-FAD2) or FAD3 (GFP-LAMP1-FAD3)] or modified versions thereof. The reporter protein (GFP-LAMP1), which is normally localized to the plasma membrane (data not shown), is ER-localized when fused to the FAD2 or FAD3 C-terminal peptide signals (upper panels). Localization to ER is abolished, however, when key residues in the FAD2 or FAD3 signals are mutated to alanines (YL for FAD2, KK for FAD3), resulting in localization of the reporter protein once again to the plasma membrane (lower panels). Bar = 10 μm. Reproduced from McCartney et al. 2004; copyright Blackwell Publishing. (C) Localization of DGAT1 and DGAT2 in distinct subdomains of the ER. Shown are immunofluorescence micrographs of suspension-cultured tobacco BY-2 cells cotransformed with DNA encoding either myc-epitope-tagged DGAT1 or GFP-tagged DGAT2 from tung. Myc-DGAT1 and GFP-DGAT2 were each localized to distinct subdomains of the ER (red and green staining patterns, respectively), suggesting that there are discrete regions of the ER associated with metabolic activities of each enzyme. The hatched box represents the portion of the cell shown at higher magnification in the panel on the right. Bar = 10 μm. Reproduced from Shockey et al. 2006; copyright American Society of Plant Biologists.

(-KxKxx; where -x- is any amino acid) that is typically found at the C-terminus of most ER-resident membrane proteins. In contrast, FAD2 proteins (as well as many diverged FAD2 proteins including tung FADX) contained a putative C-terminal aromatic-rich motif similar to a less characterized – WxxxW – motif present in only a few yeast and mammalian proteins (McCartney et al. 2004). These observations suggested that FAD2 and FAD3 proteins contain distinct retrieval motifs that are responsible for their steady-state localization in the ER. Experimental evidence in support of this premise was obtained by analyzing in onion epidermal cells the subcellular localization of various transiently expressed constructs encoding a green fluorescent protein (GFP) reporter containing the C-terminal peptides of FAD2 or FAD3 or modified versions thereof (Fig. 3B) (McCartney et al. 2004). When the critical lysine residues in the FAD3 dilysine motif were changed to alanines, the signal was no longer functional for ER retrieval, and the protein was targeted to the plasma membrane (Fig. 3B). Similarly, while GFP bearing the FAD2 C-terminal – YNNKL – sequence was localized to the ER, mutations of the critical Y and/or L residues in the FAD2 motif abolished ER localization, resulting in the reporter protein being localized at the plasma membrane (Fig. 3B). Furthermore, comprehensive mutational analysis of the novel ER retrieval motif in FAD2 indicated a consensus sequence consisting of ϕ-x-x-K/R/D/E-ϕ-COOH, where ϕ is any large, hydrophobic amino acid and –x– is any amino acid (McCartney et al. 2004).

These data support a working model whereby plant FADs are initially targeted to ER by cotranslational insertion into ER membranes and their retention in this organelle is mediated by C-terminal peptide signals that act to retrieve escaped proteins from the Golgi back to the ER. Knowledge of the novel C-terminal retrieval motif of FAD2 and diverged FAD2s subsequently revealed, through a bioinformatics search of the *Arabidopsis* proteome, that many other known and candidate ER-resident membrane proteins, including several members of the cytochrome P_{450} family, fatty acid elongases, phospholipid:DGATs (PDAT), and DGATs possess this motif and, thus, may use a similar mechanism for ER localization (McCartney et al. 2004, Shockey et al. 2006). Furthermore, recent experiments with DGAT proteins have demonstrated that these proteins are located in distinct regions or 'subdomains' of ER (Shockey et al. 2006), suggesting that that these C-terminal motifs might be involved in delivery of functionally related proteins to similar regions of ER. Addition of the C-terminal aromatic motifs of DGATs to reporter proteins, however, resulted in localization of these proteins to general ER, rather than subdomains of ER (Shockey et al. 2006), indicating that other portions of the proteins are required for their localization and/or organization into functionally distinct ER subdomains.

Regulation of FADs in ER membranes

In addition to understanding how FADs are targeted to and retained within the ER, it is important to understand how FAD activity is regulated within this subcellular compartment. The majority of evidence to date indicates that plant FADs are regulated primarily at the post-transcriptional level. For example, exposure of plants to cold temperatures often results in an increase in poly-unsaturated fatty acid content to maintain proper membrane fluidity, but FAD genes are typically not upregulated during this process (Falcone et al. 2004 and references therein). In addition, the fatty acid composition of seed storage oils can also change with temperature (Oliva et al. 2006), indicating that oil traits, as well as membrane lipid composition, can be affected by environmental conditions.

The maintenance of membrane fluidity is essential for proper cellular function, and multiple mechanisms have evolved in eukaryotic cells to help regulate FAD activity including induction/repression of gene expression, changes in mRNA stability, translational efficiency, and post-translational enzyme stability. Post-translational regulation of FADs, in particular, offers a rapid method of adjusting membrane lipid composition in response to sudden environmental changes. Studies conducted in mammals, fish, yeast and plants are beginning to define the underlying details of post-translational FAD regulation and common themes are beginning to emerge. For instance, ER-localized FADs are generally short-lived proteins, which provides a mechanism for rapidly terminating FAD activity once gene expression and/or mRNA translation is repressed (Heinemann and Ozols 2003). In addition, the half-life of FAD proteins may be modulated by environmental cues, resulting in changes in protein abundance that correlate with changes in the amount of fatty acid products produced (Dyer et al. 2001, Horiguchi et al. 2000, Tang et al. 2005).

The most extensive analysis of FAD regulation has been carried out on mammalian ER-localized stearoyl-CoA desaturase (SCD), which is a close structural relative of the FAD2 and FAD3 enzyme family from plants (although the enzyme acts upon saturated fatty acyl-CoA substrates rather than phospholipid-linked substrates, as in plants). SCD activity is regulated primarily by nutritional and hormonal cues in animals that involve changes in gene transcription and mRNA stability (Heinemann and Ozols 2003, Ntambi and Miyazaki 2004). SCD is also a short-

lived protein with a half-life of approximately 4 h (compared with most ER-resident proteins that posses half-lives of approximately 2–6 days; Arias et al. 1969). The protein also contains a *cis*-acting signal in its N-terminal region that mediates rapid protein degradation (Kato et al. 2006, Mziaut et al. 2000) (Fig. 3A). Deletion of this sequence from SCD stabilizes the protein, while attachment of the sequence to a reporter protein results in its degradation (Kato et al. 2006, Mziaut et al. 2000). Mutagenesis of this N-terminal sequence also revealed several lysine residues that were important determinants of the *cis*-acting signal (Mziaut et al. 2002), and recent experiments suggest that ubiquitination and the 26S proteasome are involved in SCD degradation (Kato et al. 2006).

Experiments with yeast SCD also support a role for the ubiquitin-proteasome pathway in FAD turnover and have provided insight to specific proteins that are involved in delivery of FADs to the proteasomal machinery (Braun et al. 2002). Surprisingly, these accessory proteins also are typically involved in the 'unfolded response' of the ER network. The ER is a major biofactory for synthesis of enzymes, and quality control mechanisms exist within the ER to ensure that only properly folded and assembled enzymes exit the ER for transport to other parts of the cell. Proteins that are improperly folded are transported out the ER into the cytosol, where they are ubiquitinated and subsequently delivered to the proteasome for degradation (Ellgaard and Helenius 2003). Mutation of several key components of this ER-associated degradation (ERAD) machinery results in an increase in yeast SCD half-life, providing a linkage between the ERAD machinery and the degradation of SCD by the proteasome (Braun et al. 2002). Taken together, the regulation of SCD activity by components of ERAD represents a novel aspect of this ER quality control mechanism, a process that has traditionally been associated with the regulation of nascent protein folding.

Evidence collected to date suggests that plant FADs are also short-lived proteins whose abundance can be modulated to regulate the amount of polyunsaturated fatty acids produced. For instance, Tang et al. (2005) expressed two closely related soybean FAD2s in yeast cells and observed a positive correlation between protein half-life and production of linoleic acid. Cultivation of FAD2-transformed yeast cells at cooler growth temperatures further increased FAD2 protein half-life and corresponding steady-state amount of protein, resulting in an increase in linoleic acid content. Through a series of mutagenesis experiments, sequences in both the N- and C-termini of the FAD2 proteins were shown to contain information that determined the steady-state amount of protein (Fig. 3A), and inclusion of proteasomal inhibitors and expression of genes in various yeast mutants defective in ubiquitin conjugation implicated the proteasomal pathway in mediating FAD degradation in these cells (Tang et al. 2005). Collectively, these results suggest that plant FAD2s are also regulated by post-translational mechanisms, and adaptation of plants to chilling temperatures, at least in part, involves an increase in FAD2 half-life corresponding to an increase in polyunsaturated fatty acid content.

Other experiments conducted with wheat root tips support a role for increased protein abundance in response of the FAD3 enzyme to cooler temperatures (Horiguchi et al. 2000). For instance, exposure of wheat roots to chilling temperatures results in a substantial increase in linolenic acid (the product of the FAD3 enzyme), despite no apparent increase in the mRNA levels of FAD3 genes. Inspection of protein amounts, however, revealed a 7.5-fold increase in FAD3 protein abundance at cold temperatures, suggesting that an increase in FAD3 steady-state protein amount was the primary mechanism for increasing the enzyme's activity. While the authors of this study could demonstrate an increase in translational efficiency of the FAD3 mRNA at cooler temperatures, the antibody signal for the protein was not sufficient to investigate any quantitative or qualitative changes in protein half-life. Notably, when plant FAD3s were expressed in yeast cells, a similar 8.5-fold increase in protein abundance at cooler temperatures was observed that correlated with increased production of linolenic acid (Dyer et al. 2001). These changes in FAD3 protein amount appear to be due to changes in the protein's half-life (our unpublished data).

Overall, experimental evidence supports the possibility that ER-localized FADs in plant cells are similar to their mammalian and yeast counterparts in that they are short-lived proteins. However, unlike these other organisms where protein abundance is also significantly influenced by strong induction or repression of gene expression, plant FADs appear to be regulated primarily at the post-transcriptional level. It also appears that both translational efficiency and modulation of protein half-life influence the steady-state amount of plant FAD proteins, thereby providing a rapid and sensitive mechanism for adjusting polyunsaturated fatty acid amounts in response to external stimuli. It will be essential to further dissect this regulatory pathway to identify specific genes involved in this regulatory process and also to determine if diverged FADs are subject to similar post-transcriptional regulatory mechanisms in plant seeds. Ultimately, this acquired knowledge of FAD regulation will not only improve our ability to rationally engineer crops for enhanced chilling tolerance but will also facilitate the optimal utilization of these enzymes for production of

specific fatty acid compositions and delivery of more stable oil traits in oilseed crops.

Catalytic plasticity of the FAD enzyme family and the importance of appropriate metabolic context

To optimize the performance of FADs and diverged FADs in engineered crops, it will be important to ensure that these enzymes are placed in the correct metabolic context to provide for efficient synthesis of the correct fatty acid product(s). There is increasing evidence that many enzyme families exhibit catalytic plasticity, where a single enzyme may produce multiple products depending on the substrates encountered (Khersonsky et al. 2006). For example, Heilmann et al. (2004) showed that redirection of a normally plastid-localized FAD to the cytosol (and vice versa) resulted in activity against non-native substrates and the production of several novel lipid products. In addition, expression of tung FADX in yeast cells resulted in not only the production of eleostearic acid but also a variety of other conjugated or desaturated fatty acid products when yeast cells were cultivated in the presence of different fatty acid substrates (Dyer et al. 2002). Notably, several of these alternative fatty acid products are detected in trace amounts in developing tung seeds (whereas eleostearic acid accounts for approximately 80% of tung oil composition), indicating that in native tung tissues, FADX is not only placed in the correct metabolic context for production of eleostearic acid but also that other enzyme activities (such as phospholipases and acyltransferases) are present to help selectively channel the nascent eleostearic acid into storage oil.

While many plants are now known to contain divergent FAD2 enzymes that are capable of producing different types of industrially important fatty acids like hydroxy, epoxy and conjugated fatty acids (Cahoon and Kinney 2005, Lee et al. 1998), almost nothing is known about the metabolic context in which these enzymes (or normal FADs) operate to produce fatty acid components destined for storage oils. In addition, because the unique types of fatty acids produced by diverged FADs are incompatible with normal cellular membranes, additional enzyme activities must be present to rapidly exclude them from biological membranes. Indeed, many plants engineered to express diverged FADs show an accumulation of the unusual fatty acid in phospholipids (Cahoon et al. 2006), which likely results in decreased flux of the fatty acid into storage oil and/or also inhibits endogenous FAD2 enzyme activity (Napier 2007). These observations suggest also that the utilization of diverged FADs for production of value-added oils in transgenic plants will require placing the enzymes in the correct metabolic context to generate the desired product. The resulting question is then how would this be accomplished? Similarly, how do plants normally ensure that enzymes such as FADs and diverged forms thereof are correctly localized within the cell/organelle, both in terms of their spatial and temporal context, and to ensure that they interact with the appropriate metabolites? In eukaryotic cells, this is generally accomplished by compartmentalization, whereby organelles, or certain subregions of organelles, contain specific collections of enzymes that are organized to carry out specific, integrated metabolic activities. Thus, as described below, it is perhaps not surprising that there appear to be specific locales within the cell, specifically at the ER, that are dedicated to oil biosynthesis.

Localization of DGATs in subdomains of ER and the potential for compartmentalization of oil biosynthesis

DGAT is an enzyme that catalyzes the committed step of oil biosynthesis by transferring a fatty acid from fatty acyl-CoA to the 3rd position of diacylglycerol to produce TAG, which subsequently accumulates in cytosolic oil bodies (Fig. 2). DGAT activity is encoded by at least three enzymes, two of which are associated with ER membranes (DGAT1 and DGAT2) and a newly identified enzyme present in the cytosol (DGAT3) (Saha et al. 2006, Shockey et al. 2006). The DGAT1 and DGAT2 proteins are encoded by two distinct gene families, with DGAT1 containing approximately 500 amino acids and 10 predicted TMDs and DGAT2 with only 320 amino acids and two TMDs (Shockey et al. 2006). There are single DGAT1 and DGAT2 genes in the tung genome and these are differentially expressed in various organs, with DGAT1 expressed in a variety of tissues in a largely constitutive manner, whereas DGAT2 was induced specifically in developing tung seeds at the onset of tung oil biosynthesis. Expression of the tung DGAT1 and DGAT2 cDNAs in yeast demonstrated that the enzymes showed different specificity toward eleostearic acid, with DGAT1 showing no preference and having a similar activity as the DGAT from yeast cells (which typically does not encounter eleostearic acid as a substrate during normal cellular metabolism), while DGAT2 showed a 5-fold preferential incorporation of eleostearic acid into TAG (Shockey et al. 2006). Collectively, these results suggest that DGAT2 may be a key player for incorporation of industrially important fatty acids, such as eleostearic acid, into storage oils of developing plant seeds.

In addition to the advances on the molecular and biochemical properties of the DGATs, other interesting

observations were made when these enzymes were characterized in terms of their subcellular localization. Specifically, transient expression of tung DGAT1 in tobacco suspension-cultured cells, followed by immunofluorescence laser-scanning confocal microscopy, revealed that DGAT1 was enriched in distinct regions of the ER (Shockey et al. 2006). Similar results were observed for DGAT2 proteins, and fluorescence recovery after photobleaching experiments demonstrated that these regions within the ER were not simply static aggregates of DGAT protein, but rather dynamic regions of the ER where proteins could move into and out. Furthermore, coexpression of both DGAT1 and DGAT2 in the same tobacco cells revealed that the subdomains occupied by the two enzymes were different (Fig. 3C) (Shockey et al. 2006), suggesting that there are distinct regions of the ER that are dedicated to the specific metabolic activities of DGAT1 and DGAT2.

There is a growing body of evidence in the literature, including from the mammalian field, that supports a role for ER subdomains in oil biosynthesis. For example, recent studies of macrophages using freeze fracture electron microscopy and immunogold labeling revealed specific regions of the ER that are enriched with proteins known to be involved in the biogenesis of oil bodies (Robenek et al. 2006). There also appears to be a close physical relationship between mammalian DGAT2 and SCD (Man et al. 2006), indicating that enzymes for fatty acid modification (SCD) and fatty acid accumulation (DGAT2) may be located in the same regions of the ER, an important prerequisite for the proper channeling the fatty acid into storage oil. While the oleic acid produced by mammalian SCD is not considered to be an unusual fatty acid, and the enzyme also acts upon fatty acyl-CoA substrates rather than the phospholipid-linked substrates used by many plant FADs, the observation that enzymes involved in a similar lipid metabolic pathway may be physically located together provides an important precedent that might extend to plants. Compartmentalization of enzyme activities could help to place enzymes such as FADX in the correct metabolic context, and additional enzyme activities, such as PDAT, phospholipases and DGAT2, could also be present to help ensure that the fatty acids produced by FADX (or other diverged desaturase) are channeled specifically into storage oil and, therefore, not retained in biological membranes where they might interfere with normal membrane function. Knowledge of the protein composition of DGAT ER subdomains should lead to the identification of additional enzyme activities that are involved in storage oil production, and the proper targeting and assembly of lipid-modifying proteins to 'storage oil domains' of the ER in transgenic plants may prove to be essential for the production of high-value industrial oils in oilseed crops.

Conclusions

Plant oils represent renewable sources of biofuels and chemicals, but engineering novel oil traits requires a better understanding of the molecular and cellular aspects of oil biosynthesis. Studies with tung FAD enzymes have provided important insight to how these proteins are targeted, assembled and regulated within ER membranes and studies with DGAT enzymes further suggests that oil biosynthesis occurs in distinct regions of the ER. Additional research is required to determine the functional significance of the DGAT domains and elucidate their protein composition, with the end goal of establishing a more complete knowledge base for the rational design of crop plants that produce high amounts of industrially important oils.

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